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Abstract
Plasma surface activation and plasma polymers deposition are promising technologies capable to modulate biologically relevant surface features of biomaterials. The purpose of this study was to evaluate the biological effects of two different surface modifications, i.e. amine (NH_2-Ti) and carboxylic/esteric (COOH/R-Ti) functionalities obtained from aminopropyltriethoxysilane (APTES) and methylmethacrylate (MMA) precursors, respectively, through an atmospheric plasma jet RF-APPJ portable equipment. The coatings were characterized by Scanning Electron Microscopy, FT-IR spectroscopy, XPS and surface energy calculations. Stability in water and after UV sterilisation were also verified. The pre-osteoblastic murine cell line MC3T3-E1 was used to perform the in-vitro tests. The treated samples showed a higher quantity of adsorbed proteins and improved osteoblast cells adhesion on the surfaces compared to the pristine titanium, in particular the COOH/R-Ti led to a nearly two-fold improvement. Cell proliferation on coated samples was initially (at 24 h) lower than on titanium control, while, at 48 h, COOH/R-Ti reached the proliferation rate of pristine titanium. Cells grown on NH_2-Ti were more tapered and elongated in shape with lower areas than on COOH/R-Ti enriched surfaces. Finally, NH_2-Ti significantly enhanced osteocalcin production, starting from 14 days, while COOH/R-Ti had this effect only from 21 days. Notably, NH_2-Ti was more efficient than COOH/R-Ti at 21 days. The amine functionality elicited the most relevant osteogenic effect in terms of osteocalcin expression, thus establishing an interesting correlation between early cell morphology and later differentiation stages. Taken together, these data encourage the use of the functionalization procedures here reported in further studies.

Keywords
titanium; surface modification; amine functionality; carboxylic/esteric functionality; in vitro cell model.

Introduction

Additive modification methods have attracted increasing interest to modulate biologically relevant surface features of biomaterials in a controlled way. Immobilized proteins and peptides have been successfully used for enhancing cell adhesion and spreading via integrins, which are an established prerequisite for the occupancy of an orthopedic implant surface [1,2]. Besides these biomimetic surface modifications aimed at exploiting essential components of the extracellular matrix, such as type I collagen, fibronectin, etc, several other types of surface functionalization (e.g. organic coatings with amine, carboxylic or hydroxyl-groups) have been and are currently being explored when designing intra-bony materials to promote tissue healing by enhancing cell functions [3]. Both surface functionalizations and bioinspired coatings show promising and comparable results: for instance osteoblasts grow equally well on titanium samples functionalized with amine and coupled with type I collagen [4].

Plasma technology has demonstrated to be effective for functionalizing titanium surfaces. In particular, vacuum plasma has been widely experimented supplying functional groups to the surface by means of treatments with reactive gases or plasma polymer deposition. In surface treatments the plasma creates reactive ions and radicals that etch, react or are grafted on the surface, while in deposition processes the plasma activates one or more monomers supplied in the atmosphere, which create radicals, thus initiating a polymerization process. Compared to the surface treatment, the deposition of the plasma polymers allows the addition of chemical functionalities, independently of the substrate features. Moreover, the deposition process, which makes use of low molecular weight monomers as precursors, allows a wide choice of functionalities [3]. Plasma polymers are either suited for the direct contact with osteoblasts [5] or applied as intermediate layers for the covalent [6] or electrostatic [7] immobilization of biologically active molecules aimed to improve the osseointegration (for instance, through an enhanced bone-titanium interface and better soft tissue integration). The most exploited functional groups in bioactive plasma polymers are amino/amide groups (-NH_2/-$CONH_2$) [5–12] and carboxylic/aldehydic groups (COOH/-CHO) [9,13–15].

Plasma assisted deposition of polymer thin films appears to be advantageous when compared to wet techniques, such as acid-etching, owing to the absence of chemical residuals on the surface, the avoidance of chemical waste and the reduced safety issues along the manufacturing process [16]. However, also plasma-deposited polymers have some drawbacks due to the need of balancing stability and functionality. In fact, the plasma polymer is characterised by a low degree of polymerization and by a not ordered 3D network since different radicals can be created in their stochastic interaction in the gas phase [17]. Therefore plasma polymers, which show a high degree of functional groups, are often characterised by small chains, possibly causing a high risk of instability in biological environment. Otherwise, more stable polymers are obtained improving the reticulation by increasing plasma density, thus leading to a higher fragmentation of the monomers and therefore to the loss of the functional groups [18]. In order to achieve stability without functionality loss, plasma copolymers are implemented by means of the combination of a monomer endowed with the desired functionality with another one capable to improve the 3D network [14,19]. Coating stability is mandatory to avoid not only the swelling in liquid environments, but also the oxidation and hydrolysis in storage in ambient air. The ageing of a coating relies on its polymerization degree and the presence of unsaturated bonds, which is universally observed for all functionalities [20]. In allylamine plasma polymer, for example, even if ageing converts the unstable functional groups into amide and oxime, the outstanding cell-surface contact with osteoblasts is maintained [21].

Although the vacuum plasma has been more studied, nowadays, the advances in atmospheric pressure plasma (APP) technology enable the implementation of various surface treatments and plasma polymer deposition at atmospheric pressure. APP treatments are of much simpler use, since they do not require complex and expensive vacuum systems and shorter processing time are used. Both amine [22,23] and carboxylic [24,25] functionalities have been obtained in dielectric barrier discharge (DBD) systems. Nevertheless, DBD systems display some geometric constrains, such as the distance between the electrodes, which limits the atmospheric processes advantages. Plasma torches offer the possibilities to treat surfaces that are not placed between the electrodes, deleting size constraint and allowing local treatments on 3D shapes. The most common technology for plasma torches is the arc discharge, which allows the introduction of functional groups [26,27], but cannot avoid a quite high processing temperature and the cathode erosion with the possibility that metal particles are deposited on the sample surface [28,29]. Nanopulse technology has been recently introduced to overcome plasma heating, as the nanosecond voltage pulse rise permits the formation of high energy reactive species, while the few tens of nanoseconds pulse length prevent the transition to arc discharge. The creation of such high energy species without heating initiates chemical reactions in the vapour phase and, therefore, the polymerization of monomers on the surfaces, keeping a high content of functional groups [30,31]. The technique has been recently applied successfully also in a plasma jet for the deposition of polyacrylic acid, even if coating stability is to be improved by copolymerization processes [32].
Radiofrequency atmospheric pressure plasma jets (RF-APPJ) represent a suitable alternative to combine room temperature processes, without electrode erosion, and power flexibility to optimise efficiency and process chemistry. In fact, RF-APPJ can control by process the growth of stable hydrophobic or hydrophilic silica-based coatings using various siloxane precursors even on heat sensitive substrates [33,34], or deposit functional groups from carboxylic acid [35], ethylene glycol [36] up to fluorocarbons [37] and diamond-like carbon [38] or even grow carbon nanotubes forests [39]. RF-APPJs have been often applied for cleaning and sterilization of dental implants, but they have also been tested for the acceleration of osseointegration [40–43], and the improvement of antibacterial properties to implants [44]. Argon and oxygen are the most used gases for these surface treatment applications [40,42–45].
The purpose of this study was to evaluate the biological effects elicited in vitro by two different surface modifications (amine and carboxylic) obtained from aminopropyltriethoxysilane (APTES) and methylmethacrylate (MMA) precursors through an RF-APPJ portable equipment in less than 1 minute of treatment time.

Material and Methods

Atmospheric pressure plasma jet

A schematic representation of the atmospheric pressure plasma complete deposition system is available in the “supplementary materials” section. The complete system is based on a patented [46] DBD-like jet design with two different couples of electrodes attached to the outer alumina tube (d_{out} = 6 mm, d_{in} = 5 mm) capacitive coupled with two power supplies working at different frequencies. The upper stream ring electrodes are spaced 1.5 mm and are coupled to a high voltage (HV) power supply up to 12 kV, working at a tuneable frequency in the range 15 – 20 kHz. The downstream couple of the electrodes consists of a ring shape electrode and of the grounded device case and is positioned at the exit of the gas flow, where the alumina tube is ending and a flux convergence is obtained with a PEEK nozzle. The latter ring shape electrode is capacitively coupled to an RF generator (27.12 MHz) over a matching network (respectively Seren R601 and Seren AT-6). Ar gas is supplied in the outer alumina tube. The thin film precursor, carried by the Ar gas passing through a bubbler at room

temperature, is fed to the source via a centre coaxial alumina capillary whose outlet is set at the end of the RF ring electrode. In laminar flow condition the design ensures that the precursor-containing gas mixture is introduced close to the plasma jet outlet and precursors decomposition is controlled and deposition inside the nozzle is reduced. The precursor capillary has a diameter small enough to allow an undisturbed development of discharge filaments alongside the inner wall of the outer tube. Both gas inlets are equipped by mass flow controllers (Bronkhorst Mass-View). The role of HV couple of electrodes is to ignite and sustain the plasma even when Ar mixtures are used as process gas or for high precursors concentrations, the maximum power output of the generator is of the order of 20 W. The role of the RF electrode is the control of the plasma density; in this case the power output of the generator is maximum 120 W. In the jet case a gas can be fluxed to cool the electrodes and to control the atmosphere close to the plasma plume, usually air or nitrogen is used.

Sample preparation

Grade 1 commercially pure titanium samples were prepared and shaped as 14 × 14 × 0.6 mm samples (l × l × h). The samples were cleaned with acetone and rinsed with isopropanol/H_2O solution 70%, then has been sterilised in ultrasound bath for 5 min in isopropanol and rinsed in Milli-Q water.

Plasma polymer deposition

Three types of specimens were attained: pristine titanium (Ti, unmodified control), titanium with amine functionalization (NH_2-Ti), titanium with carboxylic/carboxylate functionalization (COOH/R-Ti). Amine functional groups have been obtained using aminopropyltriethoxysilane (APTES, Sigma Aldrich, ≥ 98%) as precursor while methylmethacrylate (MMA, Sigma Aldrich, ≥ 99%) has been used for carboxylic/carboxylate groups. The film-forming agent has been introduced in the alumina capillary by passing 1.5 slm of Ar (Sapio 5.0 purity) as carrier gas in a bubbler system containing the liquid monomer at room temperature, while the Ar (Sapio 5.0 purity) is supplied in the outer tube inlet at flux of 4.5 slm. The HV power supply output is 8-9 kV peak-to-peak at about 17 kHz delivering about 8W for the NH_2-film deposition while less then 4W for the COOH/R- film; the RF generator at 27.12 MHz has been set to a power output of 20W for both precursors. N_2 gas (Sapio 5.0 purity) fluxed in the jet case has been used to control the atmosphere close to the plasma plume.

A representative number of samples underwent physical and chemical characterization before the biological experiments. On titanium substrates the deposition time was in both depositions 15 s for a homogeneous coating of the 14 × 14 mm area and for substrate-nozzle distance of 5 mm. For FT-IR analyses in transmission mode the coatings were deposited on silicon (100) substrates, when referred to silicon substrates samples 'Ti' is replaced by 'Si' in the nomenclature.

Microscopy

Scanning Electron Microscopy

Microstructure was studied by means of a Scanning Electron Microscope (Zeiss EVO 50, Carl Zeiss AG, Oberkochen, Germany) with Energy Dispersion Spectroscopy analyser for elemental composition analysis. To avoid contamination, the samples were washed in distilled water and rinsed thoroughly in 70% ethanol and cleaned ultrasonically for 20 min in absolute ethanol and air dried under a laminar flow hood.

X-ray Photoelectron Spectroscopy

XPS signals were collected using a SPECS (Phoibos MCD 150) X-ray photoelectron spectrometer, with Mg Kα radiation (1253.6 eV) as X-ray source, having a power of 150 W (12 mA, 12.5 kV). The spot size of the analyzed region was 7 x 20 mm. The emission of photoelectrons from the sample was analyzed at a take-off angle of 90° under UHV conditions. No charge compensation was applied during the acquisition. After collection, the binding energies were calibrated using as reference the adventitious carbon C1s peak. The accuracy of the reported binding energies (BEs) can be estimated to be ± 0.1 eV. The XPS peak areas were determined after subtraction of a background. The atomic ratio calculations were performed after normalization using the Scofield factor of each element. All spectrum processing was carried out using the Casa XPS v2.3.13 software (Casa Software Ltd.) package and Origin 7.1 (Origin Laboratory Corp.). The spectral decomposition was performed by using Gaussian-Lorentzian (70%/30%) functions, and the FWHM was fixed for each given peak.

Roughness and thickness

A stylus profiler (Alpha Test IQ from KLA-Tencor, Milipitas, California) was used to measure the surface roughness and thickness of silicon wafer coated samples. For roughness determination ten measurements of 10 mm length were conducted for each of the samples according to two amplitude parameters (R_a and R_q). R_a is the arithmetic mean of the absolute values of the surface point departures from the mean line within the sampling area. R_q (formerly called root-mean-square or RMS) is the geometric average height of roughness-component irregularities from the mean line measured within the sampling length. R_q is more sensitive to occasional highs and lows, making it a valuable complement to R_a.
Coatings thickness measurements were performed on silicon substrates by applying a scratch on the coating using a steel scalpel; the so obtained track allows a double-step height analysis that provides the searched value.

FTIR Spectrophotometry

Silicon wafer coated samples were used for transmission FT-IR spectrophotometry characterisation. Measurements were performed by using a Perkin Elmer Spectrum One spectrophotometer by performing 32 scans for each sample.

Contact angle and surface energy evaluation

The surface energy of the samples was estimated by the measurement of the static contact angle of two different liquids: water as polar probe liquid and diiodomethane as non-polar probe liquid. A FTA 1000 C Class instrument (First Ten Angstrom, Portsmouth, Virginia) was used to perform these characterizations. Briefly, a drop of the probe liquid, with a volume of 0.5 µl was poured onto the treated surfaces and a picture of the drop shape was acquired after 10 seconds of contact, by the high-resolution camera. The contact angle was determined using the data analysis software provided together with the instrumentation by fitting the sessile drop profile.
For each sample the contact angle measurements were performed three days (72h) after the sample preparation in order to reduce the influence of the plasma activation effect. For each sample and for each probe liquid (water and di-iodomethane) the contact angle measurement was repeated five times recording the related average value. The values of the two liquids were then used for calculating the surface energy value of the treated samples with its related dispersive and polar components, by applying the Owens Wendt method [47].

Protein adsorption

As reported elsewhere [48], to quantify the amount of protein adsorbed, the titanium disks were incubated in presence of Fetal Bovine Serum (FBS) in Phosphate Buffered Saline (PBS) at 2% concentration, at 37°C for 30 minutes. Subsequently, the samples were washed twice with PBS and the adsorbed protein was eluted from the disks using Tris Triton buffer (10mM Tris (pH 7.4), 100 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10% Glycerol and 0.1% SDS) for 10 minutes. Total protein amount was quantified using SERVA BCA Protein Assay Micro Kit (SERVA Electrophoresis GmbH, Heidelberg, Germany) ~~Pierce™ BCA Protein Assay Kit (Life Technologies, Carlsbad, California, USA)~~ following the manufacturer's instructions.

Cell assays

To characterize the biological response *in vitro*, the pre-osteoblastic murine cell line MC3T3-E1 (ECACC, Salisbury, UK) was used. Cells were maintained in Alpha MEM supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy), 100 U/ml penicillin, 100 µg/ml streptomycin, were passaged at subconfluency to prevent contact inhibition and were kept under a humidified atmosphere of 5% CO_2 in air, at 37°C.

Cell adhesion

Cell adhesion was evaluated on titanium samples using a 24-well plate (BD, Milan Italy) as support. Cells were detached using trypsin for 3 minutes, carefully counted and seeded at 3 × 10^3 cells/disk in 100 µl of growth medium on the disks with different roughness. The 24-well plates were kept at 37°C, 0.5% CO_2 for 10 min. Before and after fixation in 4% Paraformaldehyde in PBS for 15 min at room temperature, cells were washed two times with PBS and then stained with 1µM DAPI (Molecular Probes, Eugene, California, USA) for 15 min at 37°C to visualize cell nuclei. Images were acquired using a Nikon Eclipse T-E microscope with a 40× objective. The cell nuclei were counted using the 'Analyze particles' tool of ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Cell proliferation

MC3T3-E1 cells were plated at a density of 2500 cells/well in 24-well culture dishes and the proliferation rate was assessed by Cell Titer GLO (Promega, Milan, Italy) according to the manufacturer's protocol at 1 and 2 days.

Cell morphology and focal adhesion quantification

MC3T3-E1 cells were seeded at a concentration of 5000 cells/well in a 24-well plate. After 24 hours, cells were fixed in 4% paraformaldehyde in Phosphate Buffer Saline (PBS) and stained with Rhodamine-Phalloidin and Dapi (Life Technologies, Milan, Italy) to highlight actin network and nuclei respectively. Focal adhesions were specifically detected by an anti-Paxillin N-Term 04-581 antibody from Millipore (Merk, Darmstadt, Germany). Images were acquired with a Nikon Eclipse Ti-E microscope using different objectives: Nikon Plan 10X/0,10; Nikon Plan Fluor 40X/0,75; Nikon Plan Apo VC 60X/1,40 (Nikon Instruments, Amsterdam, Netherlands). Cell spreading and focal adhesion density were quantified with ImageJ software [49].

Osteogenic cell differentiation
To assess the osteogenic differentiation, MC3T3-E1 cells were cultured in osteogenic media by supplementing the normal culture medium with 10 mM ß-glycerophosphate and 50 ng/ml Ascorbic Acid. As a late osteogenic marker, Osteocalcin (OCN) was quantified in cell conditioned media by the use of Mouse Osteocalcin ELISA Kit (MyBioSource, Inc, San Diego, USA) following manufacturer's instructions.

Statistical analysis
Data were analysed by GraphPad Prism6 (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was repeated at least three times. Statistical analysis was performed by using the Student t-test or Mann Whitney test. A p value of <0.05 was considered significant.

Results

Morphology and topography
A view of the NH_2-Ti and COOH/R-Ti surfaces by optical and SEM microscopy is reported in Fig.1. As it can be observed, both coatings are deposited uniformly on the rough surface at the micro- and nano-scale. No particular difference between the coated and uncoated surface or between the two coatings can be detected at the microscopic examination as for morphology. The whiter nanoparticles on the titanium surfaces are already present on the surface before the coating process and were considered as defects due to the production process.
The coating thickness as measured on silicon substrates is respectively 14 ± 3 nm and 28 ± 5 nm for the amine and carboxylic groups films. The few tens of nanometers of the coating are only visible by optical microscopy by the change of colour due to refractive index of the layer introduced between titanium and air interface. Even on silicon substrates both coatings grow uniformly with no island formation at micrometre scale as it is proved by surface roughness measurements, where the average roughness (R_a) increases from 1.0 to 1.4 nm in the worst case that is within the measuring error of ±0.2nm. The Ti substrates R_a is about 300±50 nm.

FT-IR analysis
FT-IR spectra (Fig. 2A) obtained for the NH_2-Si samples show as main feature the broad absorption band between 1200 – 1000 cm^{-1} related to the Si-O-C and Si-O-Si vibrations characteristic of the siloxane network [21]. The presence of the Si-OH vibration around 950 cm^{-1} and the OH stretch band between 3500 – 3000 cm^{-1} are also expected by the plasma deposition process [50]. The deposition process protected by the nitrogen atmosphere maintains also the organic character of the precursor as it can be observed by the asymmetric stretching vibrations ν-$CH_{2,3}$ at 2980 – 2880 cm^{-1} and the deformation vibration δ-$CH_{2,3}$ at 1465 – 1375 cm^{-1} as well as the presence of nitrogen containing groups in the vibration band between 1800 – 1500 cm^{-1}. The coating presents clearly a primary amine scissor band at 1558 cm^{-1} and a band at 1660 cm^{-1} corresponding to C=N stretching vibration probably of oxime groups due to amine oxidation.
The coatings have been tested on ageing by exposure to UV light to simulate a sterilisation process and in water immersion for 1 and 2 days as in cell culture procedures (Fig. 2B). In water immersion, the intensity of all absorption bands decreases proportionally; therefore no chemical changes take place, but not fully reticulated chains are removed from the surface. The process is faster at the beginning with a reduction of the siloxane band absorption of the 50% in the first day and then it slows at about 15% the second day. Therefore, after a first removal of less bonded chains, a reticulated skeleton siloxane network remains adherent to the substrate with respectively unchanged amine and oxime moieties.
On the other side the UV exposure leads to an oxidation of the amine and oxime groups and hydrocarbon chains by the formation of amide and carboxylic acids and ketones [51].

However, this surface ageing with the presence of not only primary amine but also amides and oximes may be not detrimental to cell growth, since it was capable to enhance cells adhesion and spreading [52].
Likewise, using MMA as a starting precursor, the plasma deposition process generated a coating that maintains the carboxyl functionality. The band between 1735 – 1730 cm^{-1} corresponding to the carbonyl group is clearly visible in IR spectra and suggests the presence of both carboxylic acid (-COOH) and ester (-COOR) [32]. The ester group can mainly be associated to methyl functionalities that are present in the MMA precursor and in the coating as can be observed by the CH stretch band between 3000 – 2850 cm^{-1} and the CH deformation vibrations of CH_2 and CH_3 groups between 1480 – 1440 cm^{-1} and at 1380 cm^{-1} [20]. The three bands at 1260 cm-1, 1200 cm-1 and at 1150 cm-1 are ascribable to C-O stretch modes. These features are all present also in standard commercial poly-methylmethacrylate (PMMA). However, the marking of the plasma deposition process is visible in the weak C=C stretch vibration at 1635 cm^{-1} due to unreacted precursor and therefore to smaller chains and by the OH broad band around 3500 cm^{-1} footprint of the random reaction mechanism. These absorption bands have very low intensity in the deposited coating and the absence of an intense absorption between 1000 - 800 cm^{-1} and 3100 – 3000 cm^{-1} testifies that the aliphatic methyl groups are present as saturated structures.
Moreover, the coatings deposited by MMA precursor show very high stability in water environment with any change in intensity and in spectra features after immersion for 2 days and also after UV irradiation show only a small broadening of the aliphatic ester vibration. This broadening due to UV can be associated to the formation of ketones (1720 -1705 cm^{-1}) carboxylic acids (1725 -1700 cm^{-1}) and aldehydes (1740 -1730 cm^{-1}) [20].

XPS analysis

XPS survey analysis is depicted in Fig. 3, while results of high resolution analysis are shown in Table 1. The presence of a titanium signal can be observed on all the survey spectra, although in COOH/R-Ti and NH_2-Ti samples it is strongly reduced by the presence of the coating. In fact, the XPS sampling depth is about 5 nm, the coating thickness is about 20 nm and substrate R_a is about 300 nm, therefore the morphology can induce some shadowing in the deposition process, leading to thickness inhomogeneity and therefore preventing a full surface coverage at the nanoscale. The presence of Sn signals, which is stronger on the untreated Ti samples, can probably be addressed to a contamination of the titanium surface, which could be produced by the mechanical treatments to which the material has been subjected during the preparation of the samples. Conversely, since a Si signal is observed only for NH_2-Ti and COOH/R-Ti samples, this could be due to a contamination provided by the plasma process. The high resolution analysis has been conducted on the C1s, O1s and N1s XPS peaks, in order to investigate the bonding states on the surface after the different treatments. A considerable difference can be observed between the bonding states of C relative to the different treatments: especially the untreated Ti sample shows a much intenser contribute of the C-(C,H) band respect to the other two treatments. As for the O1s bondig states, a much stronger Ti oxide contribution is observed for the COOH/R-Ti treatment respect to the NH_2 one, and an even stronger contribution is observed for the untreated Ti sample. Analysis of the N1s peaks reveals a strong difference between the NH_2-Ti and the COOH/R-Ti treatments, since the former gives rise to a much stronger contribution of the C-NH_2 bond, while the latter produces a stronger O=C-N contribution. The intensity of the N1s peak measured on untreated Ti samples has been found too low to allow for a correct deconvolution procedure, so it has not been included in the table.

Contact angle analysis
As reported in table 2, the water contact angle measurements show a slightly improved wettability for both the functionalized samples, while there is not a significant difference between treated and not treated samples as regards the diiodomethane contact angles. Surface energy calculations confirm the increased surface polarity for both the treated samples. The dispersive component (γ_{SD}) does not change significantly, while the polar component (γ_{SP}) increases of about 10 units. A remarkable difference between NH_2-Ti and COOH/R-Ti samples is that the amino functionalized samples are positively charged, while the carboxylic functionalized samples are negatively charged, as pointed out by the work of Schroder et al. [9]

Protein adsorption

Protein adsorption assays show that the quantity of proteins adsorbed in both the treated samples was significantly higher compared to the pristine titanium control (Fig. 4). Furthermore, COOH/R-Ti resulted more efficient than NH_2-Ti.

Cell adhesion
To characterize the biological response *in vitro*, the widely diffused pre-osteoblastic murine cell line MC3T3-E1 was used [53]. Both the Plasma activated treatments significantly increased the number of adherent osteoblasts (10' of seeding) compared to the untreated titanium (Fig. 5).

Cell proliferation
As portrayed in Fig. 6, cell proliferation at 24 h was significantly lower in both the plasma activated treatments compared to the pristine titanium (control). At 48 h, the cells plated on the NH_2-Ti surface were significantly less than the control, while the condition on COOH/R-Ti did not differ from the control.

Cell morphology and focal adhesion quantification
Notwithstanding the reduced proliferation on the amine coated specimens, MC3T3-E1 cells grew properly on all the different surfaces (Fig. 7A). Fluorescent images of adherent cells at 24 h clearly show that cell mean spreading areas were significantly larger on COOH/R-Ti than on NH_2-Ti and pristine Ti (Fig. 7B), while the focal adhesion density and the AR index resulted significantly higher on NH_2-Ti (Fig. 7 C). On the whole, cells grown on amine functionalizaties are more tapered and elongated in shape with lower areas than on carboxylic/carboxylate enriched surfaces.

Osteocalcin
The osteogenic differentiation was measured by evaluating the osteocalcin release in the media at 7, 14 and 21 days (Fig. 8). NH_2-Ti significantly enhanced osteocalcin production starting from 14 days, while Ti-COOH/R had this effect only from 21 days. Notably, NH_2-Ti was more efficient than COOH/R-Ti at 21 days.

Discussion

The rapid cellular acceptance of the recipient tissue after implantation is a paramount factor dictating the success of any bone implant material, hence the interest of the functionalization processes based on the atmospheric plasma jet device here described. Starting from aminopropyltriethoxysilane (APTES) and methylmethacrylate (MMA), amine and carboxylic/esteric functionalizations were produced and chemically characterized at the interface of titanium substrates. Particularly, the efficiency of the deposition process was assessed by FT-IR spectroscopy, whilst the morphological features of the coatings were revealed through scanning electron microscopy (SEM) and the nature of the functional groups determined by high resolution XPS. Surface roughness was evaluated finding that the functionalization process does not alter the material topography in a significant way. Coatings presented a sufficient retention of functional groups and were stable in water and after UV sterilization.

The surface energy measurements demonstrated that the coated samples were more hydrophilic than pristine titanium, consistently with previously published reports [54]. As it was confirmed in the present paper, surface wettability is known to play a key role in driving protein adsorption and thereby the immediate cell adhesion dynamics [48,55]. Cell proliferation on coated samples was initially (at 24 h) lower than on titanium control, while at 48 h, COOH/R-Ti surface reached the proliferation rate of pristine titanium. On the contrary, NH_2-Ti was not so efficient as the other surfaces in promoting cell proliferation. Interestingly, as assessed by fluorescent images, NH_2-Ti altered significantly cell morphology that is a function of the dynamic interactions occurring among the cytoskeleton, cell membrane and adhesion complexes that interface with the extracellular matrix, often via the actions of regulatory signal transduction systems (Watson, 1991). Osteoblasts grown on amine functionalities were more tapered and elongated in shape with lower areas than the cells cultured on carboxylic enriched surfaces, as portrayed by the AR index. In addition, the highest focal adhesion density was found on NH_2-Ti, as evidenced through the immunofluorescent detection of phosphorylated paxillin, an actin-associated protein present in focal adhesions along with vinculin and focal adhesion kinase (FAK).

In the present study, the amine functionality elicited the most relevant osteogenic effect in terms of osteocalcin expression in later stages, thus establishing an interesting correlation between early cell morphology and later differentiation stages. Apparently and unsurprisingly, the proliferative and differentiative effects of the surface coating investigated were inversely proportional [56]. Nitrogen containing functional groups are characterized by a positive surface charge in aqueous environments and have been previously described to promote osteoblasts' growth by their beneficial interaction especially with hyaluronan, a negatively charged component of the extracellular matrix [4]. Hyaluronan may facilitate the initial interface interaction on metal surfaces that typically lack binding domains for adhesion receptors increasing the temporal gap between the early cell attachment and the advanced stages of cellular events [57–60].

According to the literature, different surface functionalizations supported stem cells differentiation also depending on the culture conditions. While promoting the osteogenesis of bone-marrow-derived mesenchymal stem cells (MSC), the NH_2-enriched surfaces showed the lowest level of viable cell adhesion under chondrogenic conditions compared to other functionalities [9]. The complexity of biological systems indeed requires several parameters besides the qualitative chemical characterization be taken into account. For instance, the density of the functional groups and their stability play a key role, as it was pointed out for the adhesion of osteoblast cells, which was optimal within a specific window of surface densities of the carboxylic groups [19].

The attempts to regulate cell fate through external cues are emerging as important methodologies in tissue engineering and regenerative medicine [61,62]. Although further in vivo experiments are suggested prior to clinical use, the functionalization procedure reported in this paper deserves consideration due to its simple use and promising outcomes.

Conclusions

In the present work, by a suitably designed APPJ, it was possible to deposit carboxylic/esteric and amine functionalized plasma polymers as in DBD systems with high deposition rate. The RF design allows to preserve the functional groups of the monomers and, at the same time, to obtain coatings that are stable to UV-based sterilization processes and in aqueous environments.

The effect of the coatings on cell growth and differentiation was tested in vitro through murine MC3T3-E1 finding that the NH_2-enriched surfaces improved cell differentiation, i.e. osteogenesis, while COOH/R functional groups enhanced cell adhesion. These data are consistent with the other literature results, although, to the authors' knowledge, this is the first report directly comparing these two different functionalizations.

The flexibility of the presented surface functionalization process, by virtue of a wide range of potential monomers in vapour or aerosol phase, offers the researchers a new tool to investigate how to regulate cell fate modulating surface chemistry. It also allows the functionalization of even complex 3D shaped materials and devices, such as dental implants, in less than 1 minute.

Aknowledgements

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Figure Legend

Figure 1. (top) COOH/R-Ti (A) and NH_2-Ti (B) surfaces are visualized per Optical Microscope in an area where part of the surface has been masked to highlight the coating presence. (bottom) COOH/R-Ti (C) and NH_2–Ti (D) surfaces are visualized per SEM at high magnification.

Figure 2. FT-IR spectra of NH_2-Si (A) and COOH/R-Si (B) samples as deposited respectively compared with the spectra after UV exposure for 35min and after water immersion for 24h and 48h.

Figure 3. XPS analysis. Survey spectra of Ti, COOH/R-Ti and NH_2-Ti surfaces.

Figure 4. Protein adsorption assay performed incubating samples with 2% FBS in PBS for 30 minutes at 37C° The asterisk (*) indicates the statistical significance vs. Ti, while the symbol (§) indicates the statistical significance vs. COOH/R-Ti using Mann Whitney test considering a p value < 0.05.

Figure 5. Cell adhesion assay at 10 minutes. The asterisk (*) indicates the statistical significance vs. Ti, while the symbol (§) indicates the statistical significance vs. COOH/R-Ti using Mann Whitney test considering a p value < 0.05.

Figure 6. Cell proliferation assay measured using CellTiter-GLO at 24 and 48 hours. Error bars represent standard error. The asterisk (*) indicates the statistical significance vs. Ti, while the symbol (§) indicates the statistical significance vs. COOH/R-Ti using Student's t test considering a p value < 0.05.

Figure 7. Panel depicting the morphological characterization performed on adherent MC3T3-E1 osteoblasts: spreading area **(A)**, AR index **(B)** and Focal Adhesion Density Quantification **(C)**. Spreading areas and AR index were determined using ImageJ software, while Focal Adhesion Density was measured by normalizing the number of focal adhesions on cell area. Error bars represent standard error. The asterisk (*) indicates the statistical significance vs. Ti, while the symbol (§) indicates the statistical significance vs. COOH-Ti using Student's t test considering a p value < 0.05. Fluorescent pictures (20 x magnification) representing MC3T3-E1 cells at 24 hours

adherent on Ti **(D)**, NH_2-Ti **(E)** and COOH/R-Ti **(F)**. The cytoskeleton is labelled red with Rhodamine-Phalloidin. Cell nuclei are stained blue with DAPI. Immunofluorescent detection of paxillin (green) to visualize focal adhesions is reproduced at 40 x magnification for Ti **(G),** NH_2-Ti and **(H)** COOH/R-Ti **(I)**. Fields are chosen to portray qualitatively cells.

Figure 8. Osteocalcin quantification. Quantification of release Osteocalcin in medium at 7, 14, 21 days, evaluated using ELISA assay. Error bars represent standard error. The asterisk (*) indicates the statistical significance vs. Ti using Student's t test considering a p value < 0.05.

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